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Vitamin D_3 Modulated Gene Expression Patterns in Human Primary Normal and Cancer Prostate Cells

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Abstract The vitamin D receptor (VDR) is a member of the steroid/retinoid receptor superfamily of nuclear receptors and has potential tumor-suppressive functions in prostate and other cancer types. Vitamin D_3 (VD_3) exerts its biological actions by binding within cells to VDR. The VDR then interacts with specific regions of the DNA in cells, and triggers changes in the activity of genes involved in cell division, cell survival, and cellular function. Using human primary cultures and the prostate cancer (PCa) cell line, ALVA-31, we examined the effects of VD_3 under different culture conditions. Complete G_0/G_1 arrest of ALVA-31 cells and ~50% inhibition of tumor stromal cell growth was observed. To determine changes in gene expression patterns related to VD_3 activity, microarray analysis was performed. More than ~20,000 genes were evaluated for twofold relative increases and decreases in expression levels. A number of the gene targets that were up- and down-regulated are related to potential mechanisms of prostatic growth regulation. These include estrogen receptor (ER), heat shock proteins: 70 and 90, Apaf1, Her-2/neu, and paxillin. Utilizing antibodies generated against these targets, we were able to confirm the changes at the protein level. These newly reported gene expression patterns provide novel information not only potential markers, but also on the genes involved in VD_3 induced apoptosis in PCa. J. Cell. Biochem. 93: 271–285, 2004.

Key words: micro-array analysis; prostate cancer; vitamin D_3; paxillin; apoptosis

Prostate cancer (PCa) initiation and progression are process involving multiple molecular alterations [Isaacs and Bova, 1998]. Genomic alterations, combined with changes in the tissue micro-environment, lead inevitably to altered levels of expression of many individual genes in tumor cells. Identification of these genes represents a critical step toward a thorough understanding of prostate carcinogenesis and improved management of PCa patients. Of particular biological and clinical interest are those genes that are consistently either overexpressed or reduced in the vast majority of PCas. Such genes and their products, besides providing possibly valuable insight into the etiology of PCa, may have important utility as diagnostic markers in this disease.

The lack of effective treatments for advanced PCa has spurred research toward the development of novel chemotherapeutic and chemopreventive methods to treat the disease. VD_3 is a member of a steroid hormone family which controls calcium homeostasis, and bone formation [DeLuca, 1997], but more recently, has been shown to have anti-proliferative properties when administered to many cancer cells lines, as well as PCa cell lines, in vitro and in animal models of the disease [Guzey et al., 2002]. In this study, we identified candidate genes involved in VD_3 activity using cDNA micro-array analysis of vitamin D receptor

Abbreviations used: Vitamin D_3, $1,25(OH)_2D_3$; ER, estrogen receptor; ERE, estrogen response element; VDR, vitamin D receptor; RXR, retinoic X receptor; AR, androgen receptor; ECM, extracellular matrix; FAK, focal adhesion kinase; PAK, paxillin activated kinases; EGFR, epithelial growth factor receptor; PCa, prostate cancer; Hsp, heat shock proteins.

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(VDR)-expressing human primary tumor stromal and metastatic ALVA-31 PCa cell lines [van Bokhoven et al., 2001] and subsequent validation at the protein level. ALVA-31 cells have been shown to have the highest VDR levels of the commonly available human PCa cell lines [van Bokhoven et al., 2001] and are considered to be a good model for screening genetic changes in the presence of VD_3. The majority of human cancers are carcinomas that, by definition, arise from epithelial cells that line glands, ducts, and surfaces of organs [Landis et al., 1998]. Consequently, the focus of research to date has been on epithelial cells, or more specifically genetic changes that occur in epithelial cells as they progress from a normal to malignant state [Olumi et al., 1999]. However, the stromal cells surrounding the tumor have a role in tumor development and progression [Hayward, 2002]. The stroma is a complex mixture of cell types, of which some traditionally have been thought to have a general supportive role, including nerves, blood vessels, smooth muscle cells are clearly crucial to the function of specific organs. In these studies, the gene expression changes induced by VD_3 on normal and tumor prostatic stromal cells was analyzed. Growth assays results confirmed previous findings that, vitamin D has an approximately 50% growth-inhibitory effect on epithelial cells at shorter exposure periods and lower dose responses, but completely inhibits their growth under extended time and higher concentrations [Peehl et al., 1994; Guzey and DeLuca, 1997; Krill et al., 1999]. In contrast, stromal cells were responded to VD_3 treatment in a different fashion. In response to VD_3 administration they undergo an approximately twofold increase in proliferation in comparison to controls [Krill et al., 1999]. In this study, our data concur with earlier reports [Peehl et al., 1994] that under robust conditions of higher VD_3 concentrations and longer time exposure primary tumor stromal cells could show some growth inhibitory effect.

Our previous studies demonstrated that VD_3 reduces expression of multiple anti-apoptotic proteins in PCa cell lines and promotes the induction of apoptosis via the mitochondrial (intrinsic) pathway. In this study, we expanded these previous analyses by including cDNA microarray data from additional human primary prostate cell lines. Furthermore, we validated the down-regulation of heat shock proteins (Hsp)-70 and bcl-2, and up-regulation of Apaf1, Hsp-90, estrogen receptor-α (ERα), Her-2/neu, and paxillin genes at the protein level. Our findings confirmed that VD_3 could be useful for inducing apoptosis of PCa cells through specific gene targets and reveal significant information regarding the mechanism of action in prostate.

MATERIALS AND METHODS

Vitamin D_3

VD_3 was obtained from Fluka (St. Louis, MO). It was prepared as 10^{-3} M stock solutions in ethanol and stored at -20°C. Stock solution concentrations were confirmed by spectroscopy (AVIV model 14DS, Lakewood, NJ), using an extinction coefficient at 220–290 nm of ε 18300 for VD_3.

Cell Culture and Growth Assays

Dr. H. Miller (Denver, CO) generously provided the human PCa cell line, ALVA-31. Normal and tumor stromal cell lines (human primary PCa cells) were isolated as described previously [Krill et al., 1997, 1999]. These cells are drived from two different patients. The normal tissue cells was confirmed histologically and came from the left posterior zone of prostate of a patient with a carcinoma in the right posterior zone with a Gleason Score of 7. The tumor tissue with a Gleason Score of 8 came from a second patient. ALVA-31 cells were maintained in humidified atmosphere with 5% CO_2 in RPMI 1640 and supplemented with 10% fetal calf serum (FCS), 1 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Irvine Scientific, Santa Ana, CA). Human primary cultures were maintained in phenol deficient RPMI 1640, penicillin, and streptomycin were replaced with 25 μg/ml gentamycin (Cellgro, Herndon, VI). Cells were cultured with VD_3, for various time periods (specifically ethanol control, labeled as 0 h, the remaining treated samples 3, 6, 12) and days (1, 2, 3, 4, and 6 days). The media was changed every 2 days and new VD_3 were added to the cultures.

Relative numbers of viable cells were determined by sodium 3′-(1-(phenylamino)-carbonyl)-3,4-tetrazolium)bis-(4-methoxy-6-nitro) (XTT) (Polysciences, Warrington, PA) dye-reduction assays [Weislow et al., 1989]. Briefly, cells were seeded at a density of approximately 2,000 cells/well in 96-well flat-bottom tissue

culture plates (Corning, Inc., Corning, NY) in 200 μl culture medium. The cells were allowed to attach for 24 h, and the medium was replaced with fresh medium containing either ethanol diluent (control) or various concentrations of VD_3. The medium containing vehicle or test compounds was renewed every 2 days during the course of experiments. After incubation, cells were processed by replacing the medium with fresh RPMI 1640 containing XTT reagent (50 μl of 0.025 mM PMS-XTT reagent/well). Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2 for approximately 4 h. Absorbance at 450 nm was read using an automated plate reader, with the microplate manager 5.0.1 program (Bio-Rad, Hercules, CA). Pilot assays demonstrated linear production of XTT substrate product for cell densities up to 40,000 cells/well, a number which was empirically determined to be within the linear-range of the assay under these culture conditions. All experiments were performed in triplicates.

Cell-Cycle Analysis

ALVA-31 and tumor stromal cells were plated and treated with hormone as above. DNA content analysis was performed by fixing and permeabilizing cells, followed by treatment with RNAse and staining with propidium iodide (PI), analyzing cells by flow cytometry [Blutt et al., 1997, 2000; Guzey et al., 2002]. The relative proportion of cells with a DNA content indicative of apoptosis (<2n), diploid G_0/G_1 cells (2n), S-phase (>2n but <4n) and G_2/M-phase (4n) was determined.

Flow Cytometry

Samples were analyzed using a Beckman Coulter Epics XL Cytometer (Miami, FL). At least 20,000 forward scatter gated events were collected per specimen. PI fluorescence was collected using linear amplification with doublet discrimination engaged. PI emissions were collected after a 620-band pass filter using the Expo 32 and Mod-Fit programs (Verity Software House, Maine).

DNA Microarray Analysis

cRNA preparation. Total RNA was extracted and purified with Qiagen RNeasy kit (Qiagen, San Diego, CA). Five micrograms of total RNA were used in the first strand cDNA synthesis with T7-d$(T)_{24}$ prime (GGCCAGTGAATTGTAATACGACTCACTA TAGGGAGGCGG-d$(T)_{24}$) by Superscript™ II (Gibco-BRL, Rockville, MD). The second strand cDNA synthesis was carried out at 16°C by adding *E. coli* DNA ligase, *E. coli* DNA polymerase I and RnaseH in the reaction. This was followed by the addition of T4 DNA polymerase to blunt the ends of newly synthesized cDNA. The cDNA was purified through phenol/chloroform and ethanol precipitation. The purified cDNA were then incubated at 37°C for 4 h in an in vitro transcription reaction to produce cRNA labeled with biotin using MEGAscript™ system (Ambion, Inc., Austin, TX).

Affymetrix chip hybridization. Fifteen to twenty micrograms of cRNA were fragmented by incubating in a buffer containing 200 mM Tris-acetate, pH 8.1, 500 mM KOAc, 150 mM MgOAc at 95°C for 35 min. The fragmented cRNAs were then hybridized with a pre-equilibrated Affymetrix chip at 45°C for 14–16 h. After the hybridization cocktails were removed, the chips were then washed in a fluidic station with low-stringency buffer (6× SSPE, 0.01% Tween-20, 0.005% antifoam) for 10 cycles (2 mixes/cycle) and stringent buffer (100 mM MES, 0.1 M NaCl, and 0.01% Tween-20) for 4 cycles (15 mixes/cycle), and stained with SAPE (Strepto-avidin Phycoerythrin). This was followed by incubation with biotinylated mouse anti-avidin antibody, and restained with SAPE. The chips were scanned in a HP ChipScanner (Affymetrix, Inc., Santa Clara, CA) to detect hybridization signals. Hybridization data from text files were imported to a Microsoft Excel spreadsheet. Genes that were reproducibly regulated at least twofold (both up and down) in all comparisons were considered significant.

Antibodies and Immunoblotting

Cell lysates were prepared using RIPA buffer (10 mM Tris, [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1% Na-deoxycholate, 0.1% SDS, 5 mM EDTA), normalized for total protein content (25 μg of protein), and subjected to SDS–PAGE using 8–12% gels, followed by electro-transfer to 0.45 μm nitrocellulose transfer membranes (Bio-Rad). Blots were incubated as described [Harlow and Lane, 1999] with primary antibodies, including 1/500 (v:v) mouse anti-70 kDa hsp monoclonal antibody (Pharmingen, San Diego, CA), 1/1,000 (v:v) mouse anti-90 kDa hsp monoclonal antibody, 1/500 (v:v) rabbit Apaf-1, 1/1,000 (v:v) mouse anti-Bcl-2 monoclonal antibody, 1/200 (v:v) mouse anti-human estrogen

receptor monoclonal antibody (Stressgen, Victoria, Canada), 1/500 (v:v) polyclonal rabbit anti-human c-erb-2 oncoprotein (DAKO, Glostrup, Denmark), (Stressgen), 1/10,000 (v:v) mouse monoclonal IgG1 paxillin (Transduction Laboratories, San Diego, CA), 1/10,000 (v:v) mouse monoclonal anti-β-actin, clone AC-74 mouse ascites fluid (Sigma, St. Louis, MO). Immuno-detection was accomplished using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Hercules, CA) and an enhanced chemiluminescence detection method (ECL) (Amersham/Pharmacia Biotechnology) with exposure to X-ray film (XAR, Eastman Kodak, Co., Rochester, NY). Protein bands were quantified by scanning densitometry (Molecular Dynamics, Personal densitometer SI, Image Quant, Version 4.1, Sunnyvale, CA), and the intensity of each band was calculated as a percentage relative to the band intensity for control.

Statistical Methods and Analysis

All experiments were performed in triplicate and mean values were presented as ± standard error. A one way analysis of variance (ANOVA) was used to statistically analyze the recorded data for XTT assays. When ANOVA indicated significant differences between treatment groups with respect to one of the sets analyzed, the Dunnett Multiple Comparisons Test [Zar, 1984] was used on that data set to ascertain where the differences occured. The software used for these analyses was Graph Pad InStat, V2.02. For all statistical tests, a P value of <0.05 was considered to be significant.

RESULTS

The growth effects of VD_3 were tested human primary prostate cultures of normal and tumor stromal cells by XTT assay, for monitoring changes in relative numbers of viable cells over time in culture. VD_3 suppressed normal stromal cells at concentrations as high as 100 nM and with approximately 15% reductions. At lower concentrations we observed growth stimulations in these cells as previously reported (Fig. 1A) [Miller et al., 1992; Krill et al., 1999]. Treatment with 10–100 nM concentrations of VD_3 suppressed the growth of tumor stromal cells by approximately 50% after a 6 day exposure (Fig. 1B). ALVA-31 was chosen as an alternative prostatic cell line for growth effect comparison to the primary stromal cells [Guzey et al., 2002].

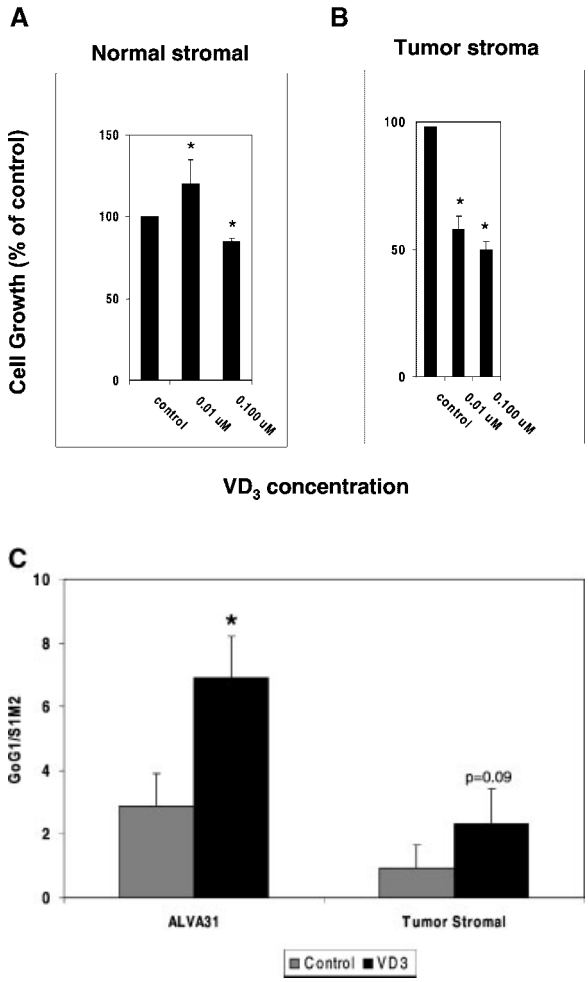

Fig. 1. Anti-proliferative effects of vitamin D_3 on normal (**A**) and tumor stromal (**B**) cell lines. Cells were plated at 2,000 cells/well in 96-well plates in 200 µl medium and cultured up to 6 days with the indicated concentrations of VD_3. Medium was changed every 2 days and replaced with fresh medium containing VD_3. The relative number of viable cells was measured using an XTT assay, expressing data as a percentage relative to control (diluent only) treated cells (mean ± SD; n = 3). Statistically significant differences ($P < 0.05$) are indicated by asterisks. **C**: Propidium iodide (PI) staining reveals a G_0G_1 population of cells after VD_3 treatment. ALVA-31 and tumor stromal cells were treated with either ethanol (vehicle, final concentration 0.1%) or 100 nM vitamin D_3 for 6 days. Cells were harvested and processed as described in Materials and Methods. Cells were recovered from culture, fixed, permeabilized, treated with RNAase, and then stained with PI. DNA content was analyzed by flow cytometry, and the relative proportion of cells with DNA contents indicative of G_0G_1 phase was determined relative to cells.

Examining the Cell-Cycle Profile of Tumor Cells Treated With Vitamin D_3

The cell-cycle profile of human primary tumor stromal cells indicate a 7% increase in G_1, 14% increase in G_0, and a reduction of 18% in the

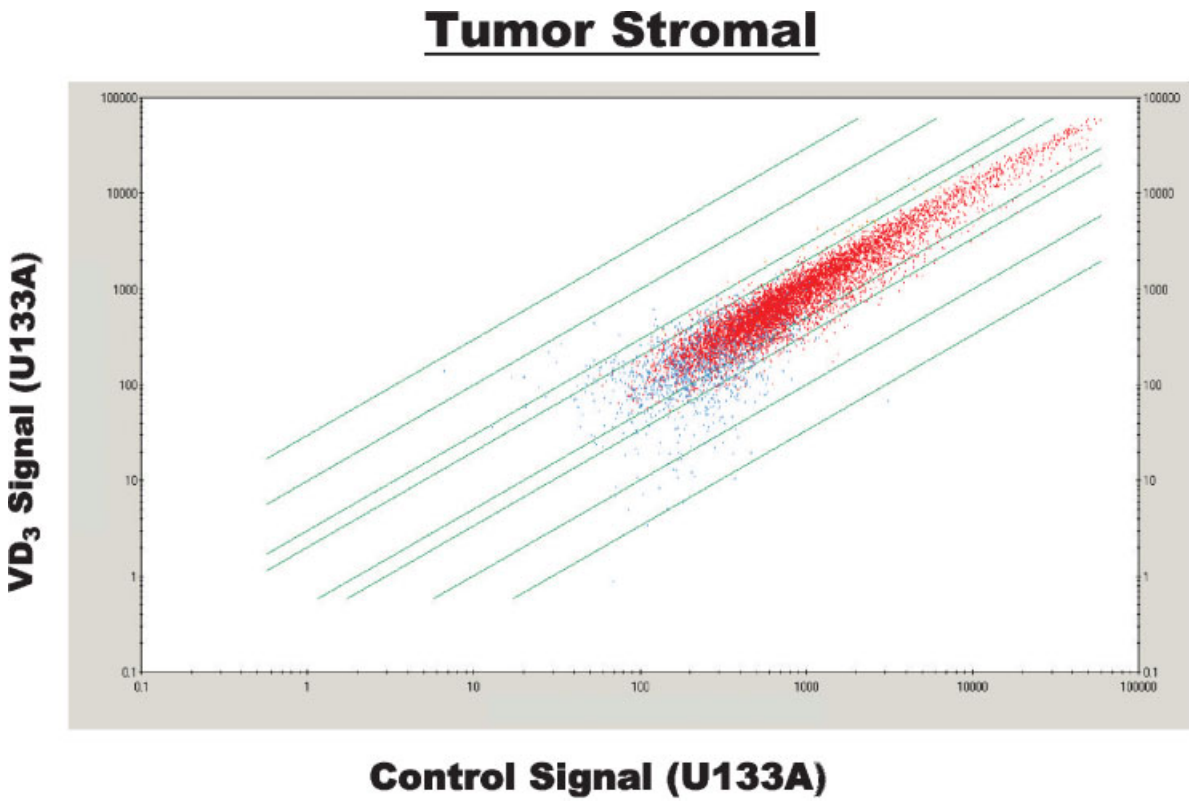

Fig. 2. The cDNA microarray analysis from RNA samples of tumor stromal cells presented as a scatter graph. More than 20,000 genes were screened by a 42K Affymetrix HuGeneFL array. Cells were treated with 50 nM VD_3 or ethanol control for 24 h and the pellets immediately rinsed and frozen at −80°C. GeneChip Expression analysis were conducted as described elsewhere [Luo et al., 2002] and a human genome U133 Set used for gene chip. The raw data analyzed by Microarray Suite, version 5.0 (MAS 5.0). Scatter graph displays points using three different colors, blue, yellow, and red to the absolute call combination of the VD_3-treated U133-signal and vehicle treated (ethanol) control-U133A-signal analyses. Briefly, yellow is represents absent, blue is marginal and red is present. The detection algorithm uses probe pair intensities to generate a detection *P* value and assign a present (P), marginal (M), or absent (A) call.

S/G_2–M phase. ALVA-31, the alternative cell line increases the number of cells in G_1 by 21%, and 7.2% in G_0. S/G_2–M phase reduced by 26% with 6 days VD_3 treatment in comparison to ethanol controls (1C). Analysis of the cell-cycle distribution after VD_3 treatment using PI staining with a large numbers of cells in G_1, and the portion of the cells contained in G_0 less than the G_1 complement of DNA, is characteristic of cells undergoing apoptosis. (Fig. 1C).

Identification of Target Genes of VD_3 by Screening of cDNA Micro-Arrays

cDNA micro-array analysis was performed to identify novel target genes of VD_3 in three different PCa cell systems: normal stromal, tumor stromal and ALVA-31.

More than 20,000 genes were screened by 42K Affymetrix HuGeneFL arrays. All three cell lines were treated with VD_3 or ethanol control for 24 h and the cell pellets immediately rinsed and frozen at −80°C. Gene Chip expression analysis was conducted as described elsewhere [Luo et al., 2002] with the human genome U133. The raw data was analyzed by Microarray Suite, version 5.0 (MAS 5.0). The cDNA microarray analysis from RNA samples of primary tumor stromal cell line is presented as scatter graph (Fig. 2). The detection algorithm uses probe pair intensities to generate a detection *P* value and assign a present (P), marginal (M), or absent (A) call. Scatter graph displays points using three different colors, blue, yellow, and red to the absolute call combination of the VD_3-treated U133-Signal and vehicle treated (ethanol) control-U133A-Signal analyses. Briefly, yellow represents absent, blue is marginal and red presents calls[1].

[1]Affymetrix, "GeneChip Expression Analysis-Data Analysis Fundamentals", Santa Clara, CA, 2002.

In order to determine genes for further analysis a cutoff of at least twofold (both up and down) in all comparisons was chosen. The selected genes have been listed for primary cells (Primary normal prostate cells and ALVA-31 microarray data not shown).

The comparison of the microarray data sets between control of tumor stromal cells to the presence of VD_3 listed in Table I. The changes in expression pattern of selected apoptotic genes was confirmed against the Oncomine database (www.oncomine.org). The genes were defined by their ID numbers according to both human Affymetrix and Oncomine microarray database.

Seven candidate genes were selected for confirmation from Table I. We grouped these genes as (a) *Hsp-70* and *-90*, *Apaf1* (b) *ERα*, (c) *Her-2/neu*, and *paxillin*. In addition to these, bcl-2, a member of the anti-apoptotic gene family, was examined.

VD_3 Treatment Induces Expression of Apaf1 Protein but Reduces Hsp-70 and Hsp-90 Protein Expression

Hsps-70, -90, and Apaf1 are critical regulators of apoptosome assembly. Hsp-70 is able to directly inhibit caspase processing by interacting with Apaf1 [Beere, 2001], to prevent the recruitment of procaspase-9 to the apoptosome [Saleh et al., 2000; Beere et al., 2000; Ozoren and El Deiry, 2002]. The protein expression of these genes was confirmed by immunoblotting in tumor stromal and ALVA-31 cells. ALVA-31 and tumor stromal cells were cultured for 0, 3, 6, 12 h and 1–4 days with VD_3, and against to their ethanol controls. Lysates were prepared on given hours and sequential days (1–4 days) and analyzed by immunoblotting, using antibodies specific for Hsps-70 and -90, and Apaf1 proteins. VD_3 induced time dependent treatment declines in the steady-state levels of Hsp-70 in ALVA-31 cells (Fig. 3A). Expression of the protein Apaf1 gradually increased and finally reached a level of 2.5-fold of the control (~150% increase) at day 4 (Fig. 3A,C). There was no change in the expression of Apaf1 at the protein level between 3–12 h nor 1–4 days of VD_3 treatment in tumor stromal cells (data not shown). Although Hsp-90 showed a slight increase with addition of VD_3 under 24 h of treatment, this level declined to ~30% of control by 3–4 days in ALVA-31 cell lines (Fig. 3A,C). In tumor stromal cells, Hsp-70 decreased >50% within 24 h, and increased to 130% at day 1. However, it gradually reduced to ~80% to control at 4 days (Fig. 3B,D). VD_3 treatment reduced Hsp-90 protein expression levels by ~30% in the first 48 h and eventually reached the same level as the controls after 3 days, and was increased twofold at 4 days in tumor stromal cells (Fig. 3B,D).

VD_3 Induces Levels of ERα

In the presence of VD_3, some nucleoproteins increase their transcriptional regulation, with the possibility that they might have additional effects on other nuclear receptors other than VDR (i.e., retinoic X receptor (RXR), AR, BAG1L [Zhao et al., 1999, 2000; Guzey et al., 2000, 2002]. For this purpose, estrogen receptor (ERα and ERβ) protein expression was quantified in both ALVA-31 and tumor stromal PCa cell lines in the presence of VD_3, as described above.

The estrogenic effects has been shown through a receptor-mediated process in human prostate tumors. There are two types of ERs, ERα and ERβ. ERα is an estrogen-dependent transcriptional factor that regulates growth, development, differentiation, and homeostasis by binding to estrogen response elements (EREs) in DNA to modulate transcription of target genes [Latil et al., 2001].

In this study, ERα is found to be increased over time in both prostate, ALVA-31 (Fig. 4A) and tumor stromal cells (Fig. 4B). Quantification of the data by scanning densitometry demonstrates an increase in the steady-state level of ERα, beginning within 1 day after treatment with VD_3, increasing to threefold in comparison to control by 2–3 days (Fig. 4C).

VD_3 Does Not Induce Levels of Her-2/neu in Primary Tumor Stromal Cells, but in ALVA-31

Her-2/neu expression has been implicated in the progression of PCa to a hormone-independent state. Her-2/neu mRNA expression has been found to be positively regulated by androgens in the androgen-dependent LNCaP cells [Zhau et al., 1992]. The transmembrane tyrosine kinase, Her-2/neu, can trigger intracellular signaling cascades such as the MAP and Akt kinase pathways. It promotes phosphorylation of the androgen receptor (AR) at multiple sites, which in the presence of low androgen concentration, results in a highly active transcriptional unit. Her-2/neu expression levels were examined upon treatment of VD_3 at different time points in comparison to the ethanol control (Fig. 5A). Her-2/neu protein expression fell to

TABLE I. Summary of Target Genes Identified Using cDNA Microarrays (Cell Line: Human Tumor Stromal)

	Gene ID[a]					
Gene names	Fold reduction [1]	Fold induction [2]	Affymetriafbohip	Oncomine	Fold status [3]	References
Bol-2	2		209364_at	Hs.79241	⇓	Welsh et al. [2001]
BAG1L	2		202387_at	Hs.41214	⇓	Luo et al. [2002]
21 OH'lase	2		214622_at	Hs.278430	⇔	LaTulippe et al. [2002]
Caspase 3	NC		202763_at	Hs.74552	⇔	LaTulippe et al. [2002]
Caspase 9	5		203984_s_at	Hs100641	⇓	Welsh et al., [2001], LaTulippe et al. [2002]
Caspase 8	NC		207688_s_at	Hs.381231	⇔	Luo et al. [2002]
VDR	2		204254_s_t	Hs.2062	⇑⇓	Dhanasekaran et al. [2001], Welsh et al., [2001], LaTulippe et al. [2002]
p21		3	203839_s_at	Hs.179665	⇑	Magee et al. [2001]
Batt		NC	208478_s_at	Hs.159428	⇑	Welsh et al. [2001]
Hsp-70		10	211015_s_at	Hs.90093	⇑	Welsh et al. [2001]
Hsp-90		5	200064_at	Hs.853561	⇑	Welsh et al. [2001]
Pasillin		2.5	211823_s_at	Hs.102497	⇑	LaTulippe et al. [2002], Luo et al. [2002]
XIAP-associated factor 1		80	206133_at	Hs.172777	⇑	Welsh et al. [2001], Krajewska et al. [2003]
24-Hydroxylase		>2	206504_at	Hs.89663	⇑⇔	Dhanasekaran et al. [2001], Welsh et al. [2001]

Selected genes have been listed for human primary tumor cells. Genes that were reproducibly regulated at least twofold (both up and down) in all comparisons were considered significant. The comparison of the microarray data sets for the selected genes to Oncomine microarray database provided.
[1,2] ligand (VD3) to vehicle (ethanol) ratio in tumor stromal cells.
[3] Oncomine, case as microarray database (www.oncomina.org). Related references are listed. These values are not ligand treated, and normal to tumor cell ratio takes unless specified.
[a]Two different gene ids' provide: 1: Human Affimetrix chip database, 2: Oncomina cancer microarray database.
⇓Decrease.
⇑Increase.
NC ⇔ not changed.

undetectable levels within 6 h of VD_3 exposure. After 3–4 days of treatment expression level increased by twofold comparison to the controls in ALVA-31 cells (Fig. 5C). The tumor stromal cells did not show any expression changes possibly because of its very low protein expression level or the lack of its importance in signaling in stromal cells.

Increases in Paxillin Protein Level in Human Primary Tumor Stromal PCa Cell Line

Focal adhesion kinase ($pp125^{FAK}$) is a non-receptor protein tyrosine kinase which transduces signals initiated through integrin activation triggered by cell/extracellular matrix (ECM) interactions [Tremblay et al., 1996a,b]. Through activation of integrins, and autophosphorylation, focal adhesion kinase (FAK) activity is increased. This in turn mobilizes numerous downstream signaling pathways. Paxillin is a signaling molecule that appears to participate in opposing cellular growth responses and is associated with proliferative signals [Salgia et al., 1999]. Endogenous paxillin levels were quantitated in tumor stromal and ALVA-31 cells. The whole cell lysates were collected at 0, 3, 6, and 12 h. The paxillin protein expression levels of the treated cells were analyzed by immunoblot and quantified by laser densitometric analysis. β-Actin was used as a loading control (Fig. 5B). An initial decrease of paxillin protein expression was observed by 6 h (3 h treatment by steroid gave identical protein expression to the control). This decrease was

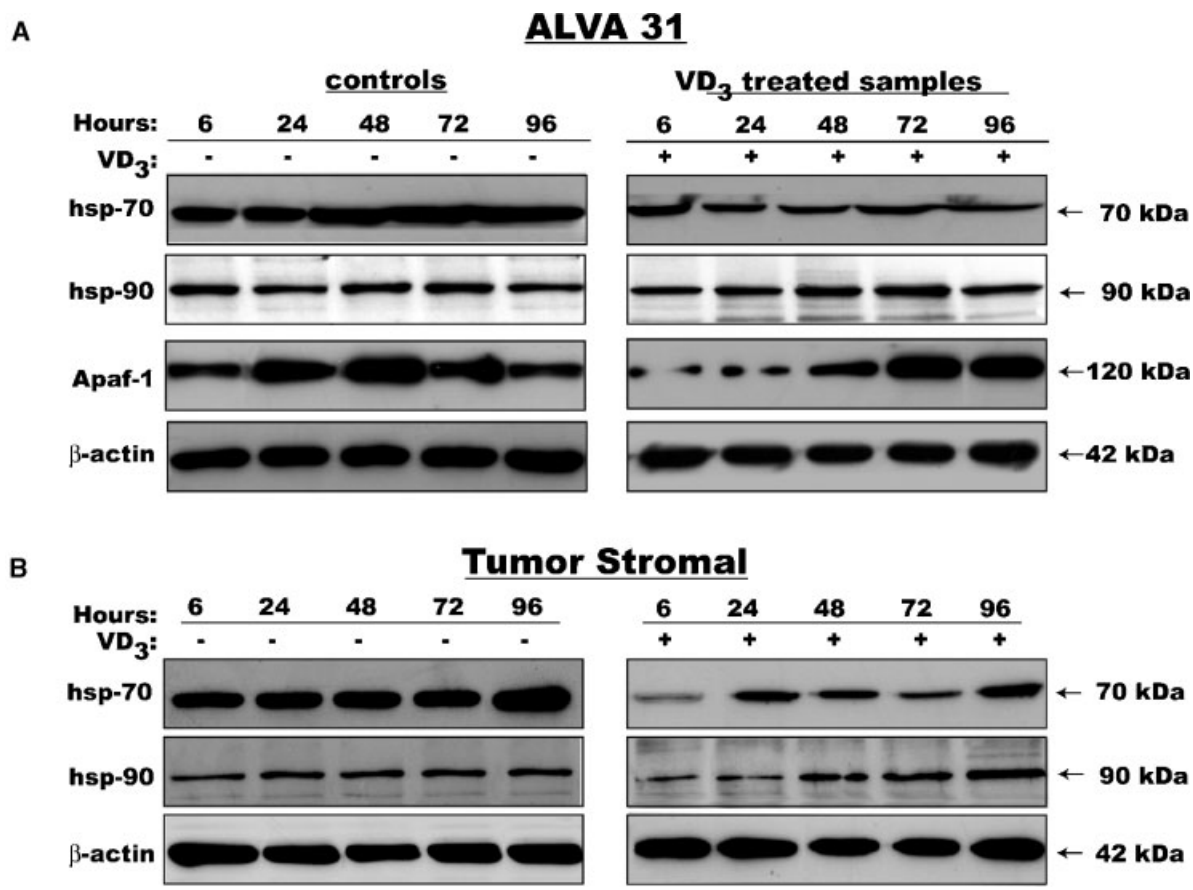

Fig. 3. VD_3 effect on expression of Hsp-70 and 90, and Apaf1 in prostate cancer (PCa) cell lines, ALVA-31 and human primary cell culture lines, tumor stromal. **A**: ALVA-31 cells were cultured for 6 h, and 1–4 days with 100 nM VD_3. Cell lysates were prepared, normalized for total protein content (25 μg/lane), and subjected to SDS–PAGE/immunoblot assay using various antibodies. All data are representative of at least three experiments. A: The time course of changes in protein levels was examined for Hsps-70 and -90, and Apaf1, using β-actin as a control in ALVA-31. **B**: Side-by-side comparisons of effects of VD_3 were performed at 6 h, and 1–4 days using antibodies recognizing Hsps-70 and -90, and Apaf1, with β-actin serving as a control in human primary tumor stromal cell line. **C**, **D**: Immunoblot data were quantified by scanning densitometry, normalized relative to β-actin loading controls, and expressed as a percentage relative to diluent-treated (control) cells. Representative data for Hsps-70 and -90, and Apaf1 are presented for ALVA-31 (C) and tumor stromal cells (D) are presented.

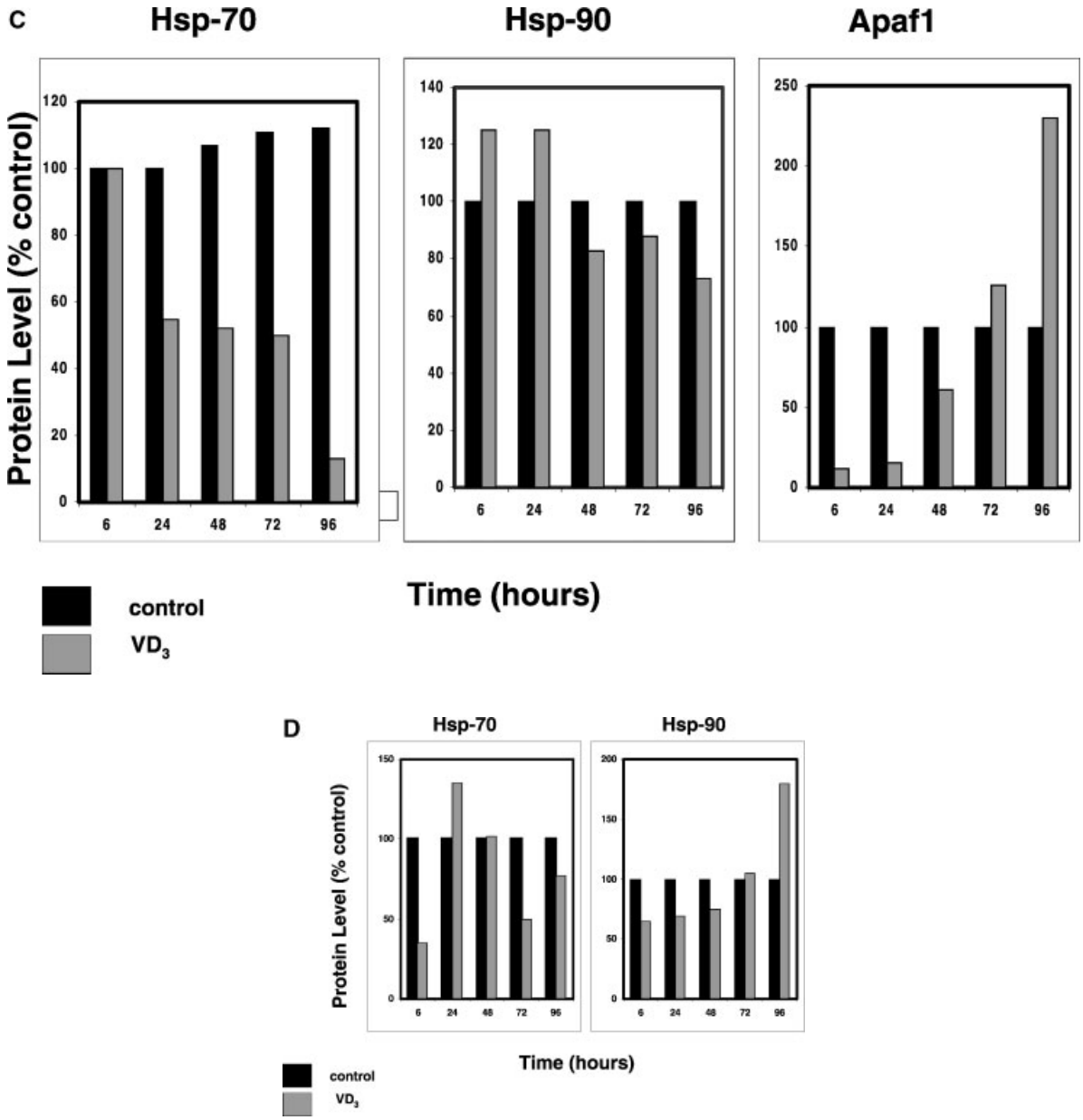

Fig. 3. (*Continued*)

>40% and continued for the first 24 h. Paxillin protein levels gradually increased to >150% (controls considered as 100%), and stayed at steady state levels until 96 h (Fig. 5D) in tumor stromal cells.

VD_3 Down-Regulates Expression of Anti-Apoptotic Bcl-2 Protein in Human Primary Tumor Stromal Cells

Bcl-2 expression in tumor stromal cells was reduced by >50% after 96 h of 100 nM VD_3 treatment (Fig. 6A,B).

DISCUSSION

The use of cDNA microarray analysis was explored to identify profiling of primary normal and tumor stromal cells, and alternatively ALVA-31 cells treated with VD_3. Further studies of selected genes in these PCa cell lines reveal a significant association of the expression levels of apoptosis related genes, *Hsp-70*, *Hsp-90*, and *Apaf1*, with VD_3 sensitivity.

The cell-cycle profile and apoptotic differences between primary and metastatic PCa

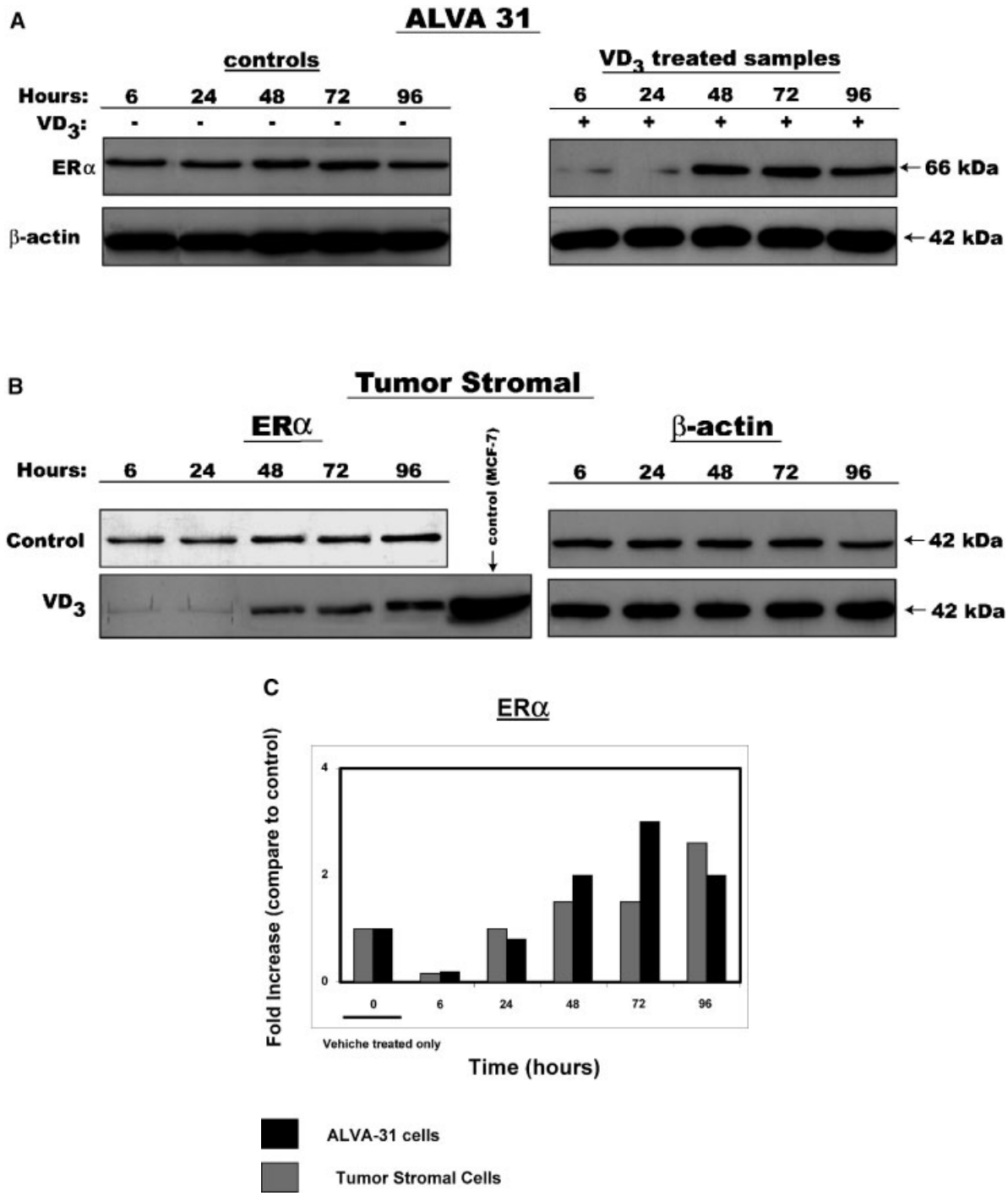

Fig. 4. VD_3 up-regulates expression of ERα in PCa cell lines. PCa cell lines ALVA-31 (**A**), and Tumor Stromal (**B**) were cultured as described in Materials and Methods. Twenty-five micrograms per lane of total protein was subjected to SDS–PAGE/immunoblot assay using anti-human ER antibodies. **C**: The immunoblot data were quantified by scanning densitometry.

lines with treatment of VD_3 could be linked to the differences in the protein expression profiles of these selected genes [Akutsu et al., 2001]. In this study, we demonstrate that these two distinct, but VDR-expressing cell lines actually contain differential protein expression profiles for cellular stress response proteins and heat shock proteins. Apoptosomes, which

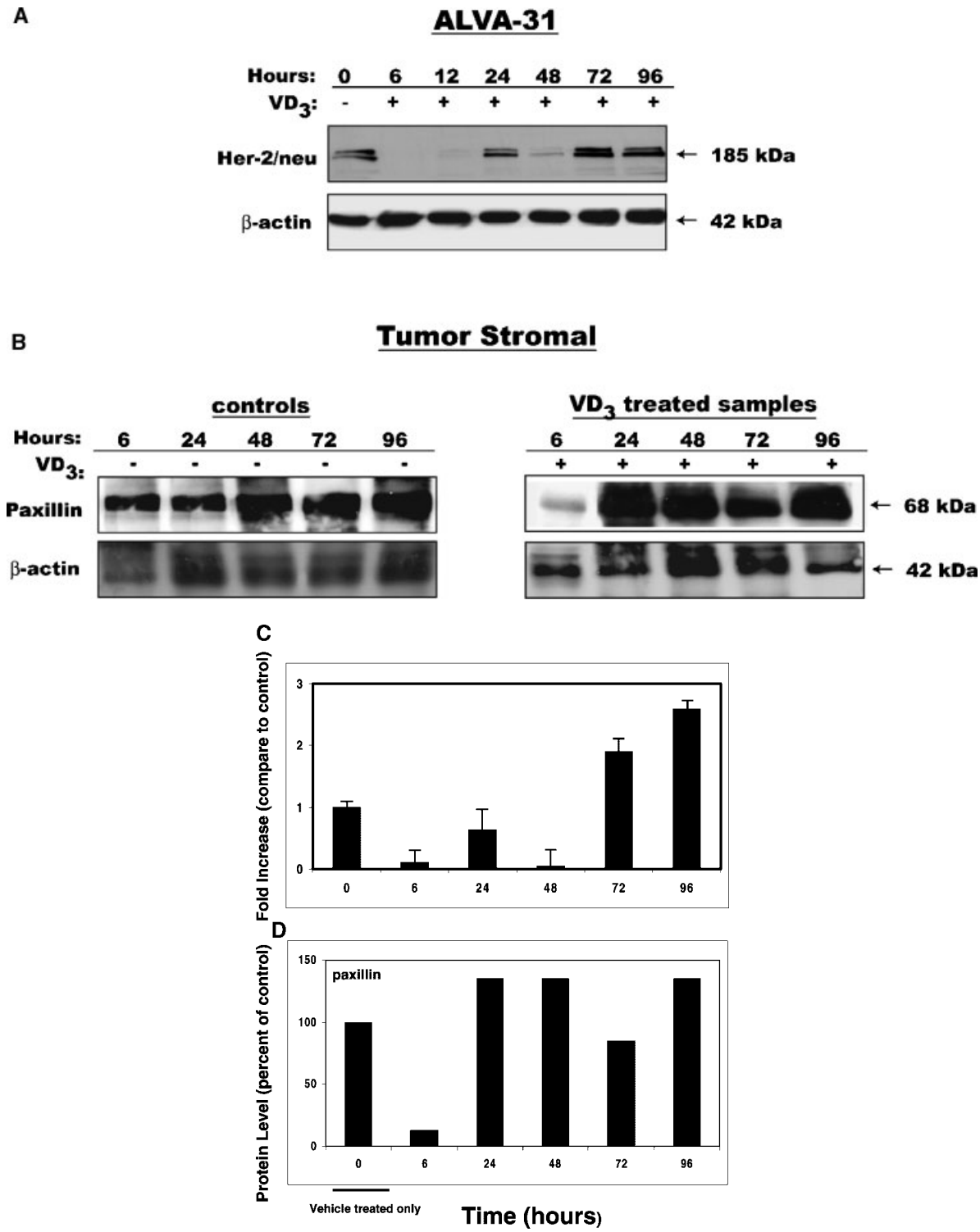

Fig. 5. VD_3 up-regulates as late response the expression of Her-2/neu and Paxillin in PCa cell lines. **A**: ALVA cells were cultured for 6 h, and 1–4 days with 100 nM VD_3. Immunoblot assay was conducted as described in Materials and Methods (12% SDS–PAGE gel) using Her-2/neu antibody. **B**: Human primary PCa cells, tumor stromal cell lysates were prepared, normalized for total protein content (25 μg/lane), and subjected to SDS–PAGE/immunoblot assay (8% gels) using antibodies recognizing paxillin followed by antibody detection using ECL methods with exposure to X-ray film. **C**: Immunoblot data were quantified by scanning densitometry, expressing results as a percentage relative to diluent-treated (control) cells. All data are representative of at least three experiments.

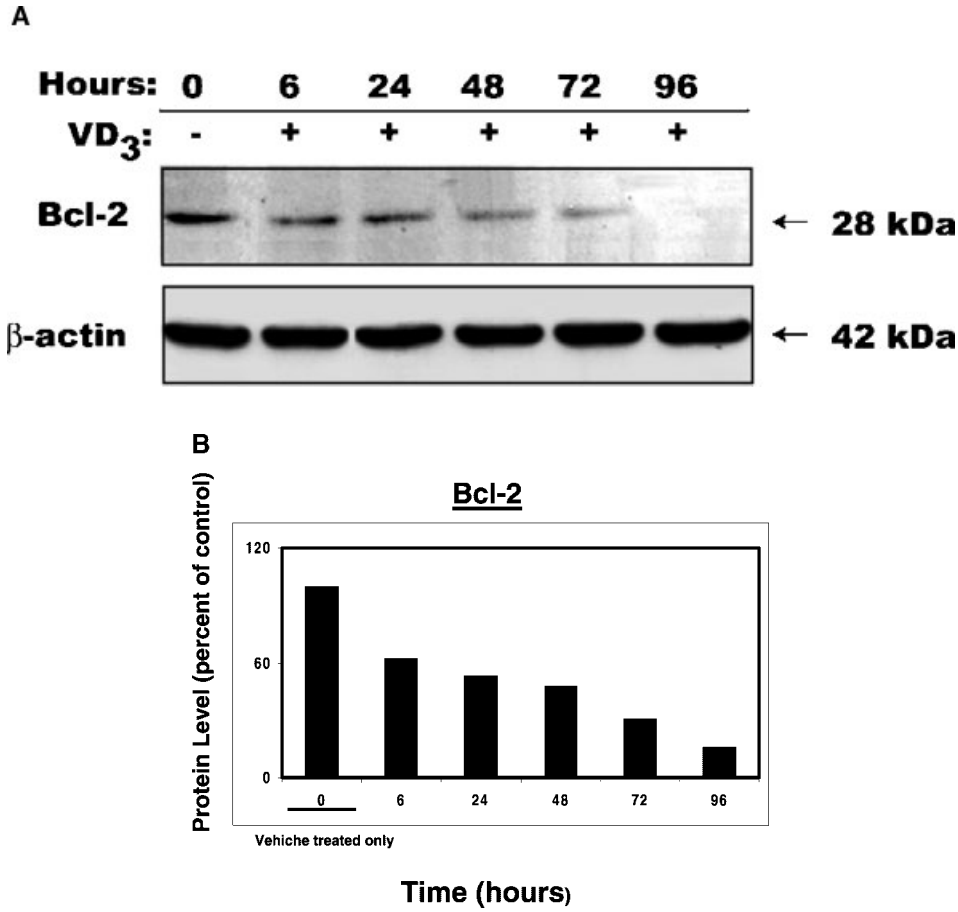

Fig. 6. VD_3 down-regulates expression of Bcl-2 antiapoptotic protein in human primary prostate culture cell lines. **A**, **B**: Tumor stromal cells were cultured for 0, 6 h, and 1–4 days with 100 nM VD_3. The time course of changes in protein levels were examined, and β-actin was used as a control.

characterize the mitochondria mediated pathway to caspase activation [Reed, 2000] are another control point for the action of VD_3. Apaf1 is involved in the activation of caspase 9 and eventually caspase 3, thus our selection of this gene provides us with a comprehensive analysis in the differences of responses of cell lines.

Previous studies have demonstrated that VD_3 induces apoptosis through the mitochondrial (intrinsic) pathway [Narvaez and Welsh, 2001; Guzey et al., 2002]. Our present data supports this finding and furthers our understanding of this potential mechanism. In ALVA-31, there is a >50% reduction in protein expression of Hsp-70. Hsp-90, despite its 25% increase during the first 24 h was gradually reduced to below 30% of control in 4 days. In this regard, the delayed time-course in which VD_3 triggers reduction in hsp-90 protein tends to suggest an indirect mechanism. Apaf1, interestingly showed a distinct picture. Its protein expression stayed the same as the untreated whole cell lysates for the first 3 h of addition of VD_3, but by 6 h this amount was reduced and stayed below the controls for the first 2 days. We initially considered the first 3 h as the earliest time point after addition of VD_3 for analysis. The cell lysates at 0 time point, which are prepared with vehicle ethanol control, gave similar results to 3 h. In that respect, we considered 6 h as the optimum starting time point to measure the VD_3 effect on cells. We acknowledge that the gene expression profiles induced or repressed by VD_3 may vary over time but consider this a critical time point for analysis. Additionally, Apaf1 protein expression was 240% by the end of 4 days. This data may support the previous findings [Saleh et al., 2000] that treatment of cells with diverse apoptotic agents dissociates the hsp-90/Apaf1 complex and relieves the inhibition of procaspase-9 activation.

Tumor stromal cells show a gradual increase in Hsp-90 protein expression in comparison to the controls for the first 3 days and then are increased approximately twofold by day 4. Apaf1 did not show any change in protein expression levels and stayed the same through the studied time points. Hsp-70 was induced and reached 130% at 24 h, but was gradually reduced to 80% of the control by day 4. This suggests that the primary tumor stromal cells have a stronger stress response, that functions to sustain survival by limiting cellular damage and accelerating recovery. Additionally, an increase in paxillin levels is possibly linked to the reduced apoptosome assembly by increased Hsps. Hsps-70 and -90 may inhibit caspase activation by interfering with the formation of a functional apoptosome [Beere, 2001]. Paxillin associates with p21 activated kinases (PAKs) indirectly, which are implicated in integrin signaling [Hashimoto et al., 2001]. This might be a reason for observing the lower G_0G_1/SM_2 ratio in tumor stromal cells in comparison to the ALVA-31 cells in the cell-cycle analysis.

VD_3 mediated down-regulation of the expression of the anti-apoptotic bcl-2 was confirmed in primary tumor stromal and ALVA-31 cells. Future studies are necessary to determine whether VD_3 induces reduction in bcl-2 through direct transcriptional mechanisms versus alternative indirect mechanisms that may involve post-transcriptional steps in gene regulation.

Steroid hormones can have profound effects on prostate tumor development and define steroid receptor expression in prostate tissues [Latil et al., 2001]. The effects of VD_3 on AR and VDR have been examined. In this study, we show that ERα protein expression level in two PCa cell lines increases with VD_3 administration. This increase was greater than fivefold compared to controls within 48 h with 100 nM VD_3 treatment in ALVA-31 cells. Tumor stromal cells had a $\geq$1.5-fold increase in protein expression level within the same parameters. Further research is necessary to enlighten the functional aspects of the changes in protein expression of ERα.

Her-2/neu is one of the most sensitive Hsp-90 client proteins, with protein expression levels reduced to an undetectable level after 6 h of VD_3 administration. However, this level increased greater than fourfold by 72 h. Her-2/neu stimulates two parallel kinase cascades, the MAP kinase and Akt pathways. Inhibition of either pathway alone fails to completely abrogate AR driven transcription. Her-2/neu induced androgen-independent survival and growth of PCa cells has been shown previously [Wen et al., 2000] in LNCaP cells. However, in the same study AR mutants also responded to Her-2/neu–Akt activation [Sasaki et al., 2002]. ALVA-31, which lack AR [Zhao et al., 1997; Yang et al., 2002] will most likely signal through a similar mechanism. Alternatively, there could be a potential strategy to target MAP kinases in PCa cells in a way that has a major impact on their survival and death [Murillo et al., 2001].

Paxillin initially showed a supressive response to 100 nM VD_3 treatment at 6 h, however was up-regulated at 1–4 days (0 h considered as whole cell lysates collected within 24 h and before addition of 3, 6, 12 h and 1–4 days intervals of VD_3 addition) in tumor stromal cells. This up-regulation reached 180% in 24 h. Tremblay et al. [1996a,b] also observed a >50-fold increase in between 18 and 40 h. However, they used epithelial cells in a quiescent state, which showed low paxillin expression. In the present study, we demonstrated possible interactions of VD_3, as a steroid ligand to the signaling molecule paxillin, which is known to be associated with FAK. The multiple cross talking signaling pathways via ERK and AKT may augment the resistance of tumor cells to the apoptosis-promoting effects [Golubovskaya et al., 2002]. Since, Hsp-70 and Hsp-90 levels were up-regulated in tumor stromal cells, this would eventually down regulate caspase 3 activation and caspase 3 dependent degradation of AKT. Reciprocally the FAK pathway would be activated and apoptosis will be prevented. The FAK C-terminal domain causes detachment and apoptosis via activation of caspase 3 and 8, and cleavage of poly (ADP-ribose) polymerase, and caspase 3 dependent degradation of AKT. Our results along with the work of others show that VD_3 activates apoptosis via the caspase 9 pathway. Although, it effects certain genes such as bcl-2 through this pathway most likely it was ineffective for paxillin for the reason described above. Additionally, it has been shown that [Golubovskaya et al., 2002], to be able to enhance apoptosis by dual inhibition of FAK and epithelial growth factor receptor (EGFR) at least in breast cancer models. Further investigations are necessary to determine the significance of the up-regulation

of paxillin in the context of androgen deprivation and its involvement to VD_3 in tumor stromal cell line.

The data provided support the hypothesis that VD_3 has an important role in regulating PCa cell growth. Screening distinct PCa cell lines, and primary tumor and normal stromal in the presence and absence of VD_3 identified novel targets for PCa. The genes that have been identified could reveal new mechanisms and new drug targets with the understanding that it might provide a novel targets for prevention and therapeutics.

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